REMARKS

Applicant submits this amendment to address the issues raised in the Office Action mailed April 7, 2005. With entry of this amendment, claims 1-60 are pending in the application. Claims 16-60 are presently withdrawn from consideration pursuant to the Restriction Requirement mailed March 7, 2000, and in view of the Response filed June 2, 2000. Thus, claims 1-15 are currently under consideration. Applicants respond to the rejections in paragraphs numbered to correspond to the Office Action.

1. Applicants confirm that claims 1-15 are pending.

2. Applicants acknowledge that the rejection of claims 2, 4, 5 and 7 under 35 U.S.C. § 103(a) has been withdrawn.

3-4. Claims 1-15 are rejected under 35 U.S.C. § 112, second paragraph because the claims do not recite the specific amino acid sequence of the mutant *Herpesviridae* thymidine kinase. This allegedly makes it difficult to determine the specific positions where the mutations occur.

Applicant submits that the specification adequately supports the locations of the mutations and they need not be specifically recited in the claims. The mutations are described in relation to the DRH and Q substrate binding domains which in turn are well defined as discussed below. Regarding the Q substrate binding domain, the specification clearly indicates at page 83, Example 10, that the codons representing residues 112-132 (second line from end) are the relevant positions. This is also described at page 18, lines 6-7.

Regarding the DRH nucleoside binding site, this site is known in the art, as described, for example, in Black, M.E., *Biochemistry* 32:11618-11626 (1993). Thus, one of skill would be familiar with the meaning and location of a DRH nucleoside binding site. As evidence, applicants draw the Examiner's attention to Wu, C.-C. et al., Biochem. J. 379:795-803 (2004), in which the term "DRH" is also employed (page 797, "Results") with reference back to the Black *Biochemistry* 32:11618 publication. Reconsideration and withdrawal of this rejection are respectfully requested.

5-6. Claims 1-15 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

At page 3, paragraph 4, the Office Action states that the specification does not provide a written description of a specific nucleotide sequence encoding a specific amino acid sequence of mutant *Herpesviridae* thymidine kinases that have mutations in Q substrate domain where the mutations result in thymidine kinase that has any increase of any biological activity or ability to phosphorylate any nucleoside analogue. The Office Action concludes that one skilled in the art cannot visualize or recognize the identity of the members of the claimed genus.

As described at page 84, lines 16-20, several hundred TK positive clones were sequenced with amino acid substitutions spanning the 20 amino acid sequence (residues 112-132, see page 83, last paragraph). Lysates from these mutants were assayed for the ability to phosphorylate thymidine, acyclovir and ganciclovir, and mutation with the Q substrate binding domain altered substrate specificity.

In order to reflect the literal language of the specification in compliance with the written description requirements, applicants have amended claim 1.

Claims 1-7 are further asserted as not complying with the written description requirements in view of the term "increase in biological activity." As discussed above, claim 1 is amended to recite the language of the specification, specifically, altering the substrate specificity. Reconsideration and withdrawal of this rejection are respectfully requested.

7. Claims 2, 4, 5 and 7 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Reconsideration and withdrawal of this rejection are respectfully requested.

The Examiner cited the Wands factors, *In re Wands*, 8 U.S.P.Q.2d 1400 (C.A.F.C. 1988). A specification is presumed to be enabling and the U.S. Patent and Trademark Office (PTO) has the burden of establishing a prima facie case of lack of enablement. *See In re Angstadt*, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976); *In re Marzocchi*, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971). To make a *prima facie* case of lack of enablement, the PTO must come forward with reasons, supported by the record as a whole, showing why the specification fails to enable one of ordinary skill in the art to make and use the claimed invention. *In re Angstadt*, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). The mere fact that some experimentation is necessary does not

negate enablement as long as undue experimentation is not required. *See* M.P.E.P. § 608.01(p).

The burden is on the PTO to establish that experimentation would be undue, *Angstadt*, 190 U.S.P.Q. at 219, taking into consideration the eight factors that are to be considered in determining whether a disclosure requires undue experimentation. *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Applicants submit that the amount of experimentation which may be required to practice the present invention does not rise to the level of being undue experimentation, as defined by the Court in *Wands*.

An important aspect of the Court's decision in *Wands* is its finding that the nature of the technology pertinent to the Wands invention (monoclonal antibody production) permitted a broad definition of the term "experiment." The Court found that an "experiment" in the monoclonal antibody art consisted of the entire attempt to make a monoclonal antibody against a particular antigen. As described by the Court, the process entailed, "immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics." 8 U.S.P.Q.2d at 1407. Thus, *Wands* supports the conclusion that in, a complex field such as monoclonal antibody production, the entire attempt to achieve the desired result, from beginning to end, constitutes one experiment.

According to the Court, repetition of this whole experiment more than once does not constitute undue experimentation. As the Court indicated, practitioners in the art would be prepared to screen negative hybridomas in order to find a hybridoma making the desired antibody. 8 U.S.P.Q.2d at 1406. Thus, the fact that some aspects of the experiment as a whole will yield negative results does not mandate a finding that the amount of experimentation to achieve a positive result is undue.

Like the production of monoclonal antibodies, the production of the nucleic acid molecules of the present claims may require some experimentation, but if viewed in the light of *Wands*, this experimentation, and the possibility of encountering negative results along the path to the positive results, is not undue. Furthermore, the present applicants provide extensive guidance to allow one of ordinary skill in the art to obtain and use nucleic acid molecules that are within the scope of the claims.

Applicants submit that one of ordinary skill in the art can construct and use nucleic acid molecules, for example, as described in the specification at pages 83-84 (Example 10). Such tests would not constitute "undue" experimentation within the scope of *Wands*, as discussed in detail below.

The inventors have, for the first time, produced TK mutants with alterations in the Q substrate binding domain. Applying this information to the eight *Wands* factors, one of skill in the art would conclude that undue experimentation would not be required to practice the claimed invention.

a. *Nature of the invention and breadth of the claims.* The invention relates to mutagenesis of TK polynucleotides to yield mutations in codons corresponding to residues 112-132 (the Q substrate region) to carry out mutagenesis, as described adequately throughout the application, and particularly in Example 10 at pages 83-84. The nature of the invention is such that it is well-known to those of ordinary skill in the art. The court in *Wands* stated that the nature of monoclonal antibody technology is that it involves screening, including screening of negative samples (in that case, hybridomas). The number of potentially negative samples was not viewed as a determining factor in reaching a finding of undue experimentation (page 8 U.S.P.Q.2d at 1406-1407). According to the present application, the method resulted in several hundred clones which were assayed, and they showed that mutation in the Q substrate domain altered substrate specificity.

b. *Amount of direction or guidance provided.* The specification provides clear directions for performing the experimentation, and cites to published scientific articles for details not mentioned in the specification. For example, Black et al., *P.N.A.S.* 93:3525-3529 (1996), is cited for a vector used in the Example 10 method. Similarly, the *Wands* court found that the starting material was available to the public (as is the material used in the present application) and the patent at issue in *Wands* provided a detailed description of the methods, which included use of a commercially available kit. (8 U.S.P.Q.2d at 1404, 1405).

c. *Presence of absence of working examples.* The specification describes production of hundreds of positive clones that exhibited altered specificity in the Q substrate region at page 84, first full paragraph.

d. *The predictability or unpredictability of the art.* In *Wands*, the Court noted that the cell fusion technique was well known to those of ordinary skill in the art, and that there was no indication that the fusion step should be more difficult or unreliable for the antigen in question (HBsAg) than for other antigens. The Examiner has provided no evidence that the mutagenesis and substrate specificity testing steps would be "more difficult or unreliable" (8 U.S.P.Q.2d at 1406) than for a comparable protein.

e. *The relative skill of those in the art.* The level of skill in the art is deemed to be high. Those of skill in this art are highly skilled and would be competent at designing and performing, or directing the performance of, the procedures of factors (c) and (d) above. The *Wands* court found that the level of skill in the monoclonal antibody art was high at the time the application was filed, but, importantly, the court found that development of skill in performing specific experiments relevant to the art did not preclude enablement. Specifically, the court stated that initial failures occurred as the inventors learned to fuse cells, and "[o]nce they became skilled in the art, they invariably obtained numerous hybridomas . . ." that met the claim limitations. (8 U.S.P.Q.2d at 1406). By analogy, it would not defeat enablement for one of skill in the art of mutagenesis to learn and become proficient in techniques for practicing the present invention.

f. *Quantity of experimentation necessary.* To determine if a method falls within the scope of the claims, the only experimentation required is the performance of known substrate phosphorylation assays, to determine if substrate specificity was altered. These procedures are routine and would not have to be done repeatedly before a definitive result was obtained. Because the inventors and the art provide means for conducting assays within the claim scope, this factor is met, for example, by the provision of hundreds of TK positive clones with Q substrate region mutations for assay of substrate specificity.

The *Wands* court found that practitioners in the art are prepared to screen negative hybridomas to find one that made the desired antibody. (U.S.P.Q.2d at 1406.) The court further stated that an "experiment" was not simply the screening of a simple hybridoma, but instead was the entire attempt to make a monoclonal antibody against a particular antigen. This process included immunizing animals, fusing lymphocytes from

the immunized animals to make hybridomas, cloning the hybridomas, and screen the antibodies produced by the hybridomas. (U.S.P.Q.2d at 1406).

By analogy, a single experiment in the present art could include producing hundreds of Q substrate mutant clones, and assaying for substrate specificity. Encountering negative results would not mean that undue experimentation is involved, according to *Wands*.

g. *The state of the prior art.* The prior art provides the methods and materials needed to apply the methods of factor (d) above to the particular region encoding the Q substrate binding domain. The *Wands* court found that "all the methods needed to practice the invention were well-known." (8 U.S.P.Q.2d at 1406). Similarly, the methods of constructing the mutants, and measuring substrate specificity, are well known.

8-9. Claims 1, 3, 6 and 8-11 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Munir et al., *J. Biol. Chem.* 267:6584-89 (1992) in view of Graham et al., *GenBank Accession No.* X03764 (Sept. 12, 1993); Kit et al., *GenBank Accession No.* X01712 J02225 (Sept. 12, 1993); Drake et al., *Antiviral Res.* 35:177-85 (1997); Waldman et al., *J. Biol. Chem.* 258:11571-75 (1983); Munch-Petersen et al., *J. Biol. Chem.* 266:9032-38 (1991); Balasubramaniam et al., *J. Gen. Virol.* 71:2979-87 (1990); Brown et al., *Nat. Struct. Biol.* 2:876-81 (1995); and Donarian et al., *Gene Therapy* 2:235-44 (1995).

The Office Action states that Balasubramaniam and Brown teach that the Q substrate binding domain and the DRH binding domain are important in nucleoside binding, that one of skill would use the random mutagenesis method of Munir to randomly mutate the codon, and that mutants having increased activity towards prodrugs would be expected to be more effective in cancer treatment when used in gene therapy as taught by Donarian et al.

The Examiner's suggestion to mutate the domains of Balasubramaniam and Brown (Q substrate and DRH binding domain) rises to the level of "obvious to try" but fails to meet the "expectation of success" standard required for an obviousness rejection. The random mutagenesis method of Munir fails to teach the codons to be mutated in order to achieve applicant's claimed invention, which is an isolated nucleic

acid molecule in which the at least one mutation in the Q substrate domain alters the substrate specificity of the thymidine kinase. Until the experiment was performed on the resulting thymidine kinase mutants assayed, one of skill, reading the references without benefit of hindsight based on applicant's teaching, would not have had the requisite expectation of success. The other references fail to remedy the deficiencies.

In view of the present arguments in favor of the non-obviousness of claims 1, 3, 6 and 8-11 over Munir in view of the cited secondary references, applicant submit that each of these claims patentable under 35 U.S.C. § 103 and respectfully requests withdrawal of the present ground for rejection.

Claims 12-15 stand rejected under 35 U.S.C. § 103 as allegedly unpatentable over Esandi et al., *Gene Ther* 4:280-87 (1997) in view of Munir, Graham, Kit, and Donarian.

The Examiner states that the arguments filed on November 29, 2000 were considered, but it would have been obvious for one of ordinary skill to make an expression vector comprising a promoter operable linked to the claimed nucleic acid encoding the claimed *Herpesviridae* thymidine kinase by inserting the mutated DNA encoding mutant thymidine kinase into the expression vector of Esandi et al. "in order to express thymidine kinase mutants in cancer cells of specific tissue origin which is expected to be effective in the treatment of cancer when these mutants are used in gene therapy as taught by Donarian et al." (Office Action page 7, lines 4-7).

The Examiner's suggestion to insert the mutated DNA into the expression vector of Esandi et al. rises to the level of "obvious to try" but fails to meet the "expectation of success" standard required for an obviousness rejection. In particular, the Examiner states that there is an expectation of effectiveness. However, the random mutagenesis method of Munir fails to teach the codons to be mutated in order to achieve applicant's claimed invention, which is an isolated nucleic acid molecule in which the at least one mutation in the Q substrate domain alters the substrate specificity of the thymidine kinase. Until the experiment was performed on the resulting thymidine kinase mutants assayed, one of skill, reading the references without benefit of hindsight based on applicant's teaching, would not have had the requisite expectation of success. The other references fail to remedy the deficiencies.

If additional fees are believed necessary, the Commissioner is further authorized to charge any deficiency or credit any overpayment to Deposit Account No. 04-0258.

In view of the above claim amendments and remarks, Applicant submits that the claims are now in condition for allowance and requests that the Examiner issue a Notice to that effect.

If questions remain regarding this application, the Examiner is invited to contact the undersigned at (206) 628-7650.

Respectfully submitted,
Margaret E. Black
DAVIS WRIGHT TREMAINE LLP

By _____
Jane E. R. Potter
Registration No. 33,332

2600 Century Square
1501 Fourth Avenue
Seattle, WA 98101-1688
Phone: (206) 628-7650
Facsimile: (206) 628-7699

MAY 3 0 2006
PATENT & TRADEMARK OFFICE

Biochem. J. (2004) 379, 795–803 (Printed in Great Britain)

Identification and characterization of the conserved nucleoside-binding sites in the Epstein–Barr virus thymidine kinase

Chung-Chun WU*, Min-Che CHEN*, Ya-Ru CHANG*, Tsuey-Ying HSU*[1] and Jen-Yang CHEN*†[1]

*Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Jen-Ai Road, Taipei 100, Taiwan, Republic of China, and †Extramural Research Affairs Department, National Health Research Institutes, Min-Chuan East Road, Taipei 114, Taiwan, Republic of China

Thymidine kinase (TK), encoded by EBV (Epstein–Barr virus), is an attractive target for antiviral therapy and provides a novel approach to the treatment of EBV-associated malignancies. Despite the extensive use of nucleoside analogues for the treatment of viral infections and cancer, the structure–function relationship of EBV TK has been addressed rarely. In the absence of any structural information, we sought to identify and elucidate the functional roles of amino acids in the nucleoside-binding site using site-directed mutagenesis. Through alignment with other human herpesviral TK protein sequences, we predicted that certain conserved regions comprise the nucleoside-binding site of EBV TK and, through site-directed mutagenesis, showed significant changes in activity and binding affinity for thymidine of site 3 (-DRH-) and 4 (-VFP-) mutants. For site 3, only mutants D392E ($Asp^{392} \rightarrow Glu$) and R393H retain activity, indicating that a negative charge is important for Asp^{392} and a positive charge is required for Arg^{393}. The increased binding affinities of these two mutants for 3′-deoxy-2′,3′-didehydrothymidine suggest that the two residues are also important for substrate selection. Interestingly, the changed metal-ion usage pattern of D392E reveals that Asp^{392} plays multiple roles in this region. His^{394} cannot be compensated by other amino acids, also indicating a crucial role. In site 4, the F402Y mutant retains full activity; however, F402S retains only 60 % relative activity. Strikingly, when Phe^{402} is substituted with serine residue, the original preferred pyrimidine substrates, such as 3′-azido-3′-deoxythymidine, iododeoxyuridine and β-L-5-iododioxolane uracil (L-form substrate), have decreased competitiveness with thymidine, suggesting that Phe^{402} plays a crucial role in substrate specificity and that the aromatic ring is important for function.

Key words: Epstein–Barr virus, nucleoside-binding sites, thymidine kinase.

INTRODUCTION

Thymidine kinase (TK) is an enzyme that catalyses the transfer of the γ-phospho group of ATP to thymidine to generate dTMP in the salvage pathway of pyrimidine synthesis. dTMP is phosphorylated further by a cellular enzyme to dTTP, which serves as a substrate for DNA polymerase during DNA replication. Herpesviral TK phosphorylates a broad range of nucleoside analogues [1], and serves as an important target for antiviral therapy. The TK enzymes convert nucleoside analogues, such as ACV (acyclovir) [2] and GCV (ganciclovir) [3] to their monophosphate form. Cellular enzymes then phosphorylate the prodrug to the active nucleoside triphosphate. The viral DNA polymerase incorporates the metabolites preferentially into the elongating viral DNA, resulting in DNA-chain termination [4,5]. Recently, the herpesviral TK, such as those of HSV (herpes simplex virus), VZV (Varicella Zoster virus), equine herpes virus 4 and EBV (Epstein–Barr virus) [6–9] also have been found to be useful suicide genes in gene therapy. The genes are introduced into tumour cells, which, subsequently, can be killed selectively by the appropriate nucleoside analogues. Much effort has been put into improving the selective killing effect by synthesizing new prodrugs [10] and constructing TK mutants [11].

EBV, a member of the herpesviridae family, is a ubiquitous virus that infects most of the human population during early life. EBV has been shown to cause infectious mononucleosis [12–14], fatal acute infectious mononucleosis, X-linked lymphoproliferative syndrome and oral hairy leukoplakia [15–17]. EBV also has a close association with several types of malignancies, e.g. African Burkitt's lymphoma [18,19], nasopharyngeal carcinoma [20], Hodgkin's disease and T-cell lymphoma [21]. Anti-viral approaches have been developed based on the complex relationship between EBV and these diseases. EBV TK, an early lytic protein encoded by the BXLF1 open reading frame [22,23], plays a major role in most of these anti-EBV strategies. Diseases involving lytic EBV infection, such as HIV-oral hairy leukoplakia, regress when treated with nucleoside analogues [15]. Recently, a new virus-specific therapy, involving induction of EBV TK by concomitant antiviral nucleoside treatment, was developed by Mentzer and co-workers [24–26], who conducted a clinical trial of the EBV-associated post-transplant lymphoproliferative disease. The expression of TK was increased by arginine butyrate, and GCV treatment caused regression of the tumour cells. Therefore a better understanding of EBV TK should permit the development of new approaches and new drugs for the treatment of EBV-associated diseases. However, unlike for the TK of α-herpesvirus, the characterization of EBV TK has been relatively slow because of the lack of a cell-culture system, which allows lytic viral replication.

EBV TK shares protein sequence and functional homology with HSV-TK but has an additional 243 amino acids at its N-terminus. The function of the N-terminal domain is unknown, but this hydrophilic region has strong antigenicity and has been shown to react with sera from nasopharyngeal carcinoma patients (P. Yeh, J.-Y.

Abbreviations used: ACV, acyclovir; AZT, 3′-azido-3′-deoxythymidine; D4T, 3′-deoxy-2′,3′-didehydrothymidine; DDI, dideoxyinosine; DTT, dithiothreitol; EBV, Epstein–Barr virus; L-FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil; GCV, ganciclovir; HHV-8, human herpes virus 8; HSV, herpes simplex virus; IdU, iododeoxyuridine; L-I-OddU, β-L-5-iododioxolane uracil; TK, thymidine kinase; VZV, Varicella Zoster virus.

[1] Correspondence may be addressed to either of the authors (e-mail tyhsu@ha.mc.ntu.edu.tw or cjy@nhri.org.tw).

© 2004 Biochemical Society

Chen and T.-Y. Hsu, unpublished work). A biochemical study revealed that EBV TK has K_m values of 22 μM for thymidine and 25 μM for ATP [27]. Substrate specificity has also been determined by several groups [27,28]. However, the structure–function relationship of EBV TK has been addressed rarely. In our previous study, we have shown that the C-terminus of EBV TK is important for its activity [29]. In the present study, we sought to identify and characterize the nucleoside-binding sites of the enzyme.

Multiple alignments reveal six highly conserved sites in the amino acid sequences of 12 herpesviral TKs [30]. Sites 1, 3 and 4 are supposed to be involved in substrate binding. HSV-1 TK has three substrate-binding sites, site 1 for ATP and sites 3 and 4 for thymidine binding, which have been confirmed by mutagenetic methods and structural analysis [31–36]. Thymidine-binding sites are also the regions that are responsible for binding to various nucleoside analogues. Attempts to engineer the thymidine-binding sites to modify the substrate specificities of viral TKs for clinical application have been reported [11]. In this study, the EBV TK nucleoside-binding sites were defined and the biochemical roles of amino acids in these regions were characterized.

EXPERIMENTAL

Chemicals

Nucleoside analogues, thymidine, ATP, phosphocreatine, creatine kinase, albumin, sodium fluoride, DTT (dithiothreitol) and metal chloride were purchased from Sigma. L-FMAU (2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil) and L-I-OddU (β-L-5-iododioxolane uracil) were gifts from Y. C. Cheng (Department of Pharmacology, Yale University School of Medicine, New Haven, CT, U.S.A.) [10]. [^{3}H]Thymidine was obtained from NEN (Boston, MA, U.S.A.).

Site-directed mutagenesis

Mutants encoding proteins with single amino acid substitutions were generated by recombinant PCR. The template for PCR was the plasmid pET-TKB1B, which encodes a full-length, 607-amino-acid TK protein [37]. A mutated complementary set of primers was designed for each specific wild-type residue (Table 1). Two PCRs were set up at the same time, one with a mutated antisense primer and the *Bam*HI primer, the other with the complementary mutated sense primer and the *Hin*dIII primer. These two PCR products were purified by electrophoresis, mixed in a 1:1 molar ratio in PCR buffer containing 10 mM Tris/HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 400 μM dNTP and 2 units of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland), then annealed and extended with one cycle at 95 °C for 5 min, one at 52 °C for 5 min and another at 72 °C for 10 min without primers. The *Bam*HI and *Hin*dIII primers were then added to the reaction and a further 25 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min were performed. The resulting products were digested with *Bam*HI and *Hin*dIII and ligated to the corresponding sites of the pRsetA vector (Invitrogen, Carlsbad, CA, U.S.A.) to obtain recombinant plasmids with point mutations in the EBV TK open reading frame. The sequences of all mutants were confirmed by DNA sequencing (ABI PRISM™ dye terminator cycle sequencing kits).

Expression of the TK protein

The recombinant plasmids obtained were used to transform the *Escherichia coli* strain BL21(DE3)pLysS [29]. One colony was seeded to Luria–Bertani broth containing 200 μg/ml ampicillin and 25 μg/ml chloramphenicol and grown until a value of 0.4–0.6 was reached for A_{600}. The culture was treated with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) for 2.5 h at 25 °C with shaking. The bacterial suspension was centrifuged and lysed with 1/50 volume of lysis buffer [0.5 M NaCl/20 mM Tris/HCl, pH 7.9/10 % (v/v) Nonidet P40], 1 mM PMSF (Sigma) and 200 μg/ml lysozyme (Sigma). After sonication (Ultrasonic, up400A), the lysate was used for Western-blot analysis and other assays or stored at − 70 °C.

Table 1 Primers used in the construction of mutants with a single amino acid change

Mutants	Sense primer (5′ → 3′)
D392E	GATTTTGCATGAACGCCATTTGC
D392H	GATTTTGCATCATCGCCATTTGC
D392N	GATTTTGCATAATCGCCATTTGC
R393Q	GATTTTGCATGATCAGCATTTGC
R393L	GATTTTGCATGATCTGCATTTGC
R393H	GATTTTGCATGATCATCATTTGC
R393E	GATTTTGCATGATGAGCATTTGC
H394D	GCATGATCGCGATTTGCTGTCGG
H394F	GCATGATCGCTTTTTGCTGTCGG
H394K	GCATGATCGCAAGTTGCTGTCGG
H394L	GCATGATCGCCTTTTGCTGTCGG
H394N	GCATGATCGCAATTTGCTGTCGG
V401C	GGCCTCCGTGTGTTTCCCTCTAA
V401L	GGCCTCCGTGCTTTTCCCTCTAA
V401S	GGCCTCCGTGTTCTTTCCCTCTAA
F402S	CTCCGTGGTTTCCCCTCTAATGC
F402Y	CTCCGTGGTTTACCCTCTAATGC

TK activity assay

The TK activity assay was performed as described previously [38]. Crude lysate (10 μl) was mixed with 75 μl of reaction mixture containing 0.16 M Tris/HCl (pH 7.5), 0.14 mM albumin, 12.6 mM phosphocreatine, 11.2 units/ml creatine kinase, 120 μM thymidine, 2.35 μM [^{3}H]thymidine (6.7 Ci/mmol), 2.4 mM ATP, 2.4 mM $MgCl_2$, 9 mM NaF and 1.9 mM DTT. The mixture was incubated at 37 °C for 30 min. A 50 μl sample was spotted on to a Whatman DE-81 filter, washed twice with alcohol and dried. The radioactivity on the filter was measured in a liquid-scintillation counter (LS-6000; Beckman, Fullerton, CA, U.S.A.). The relative-specific activity was calculated as the value of TK activity subtracted from the value of the vector control, which was almost identical with the water control, and normalized with the TK concentration calculated from enhanced chemiluminescence (ECL®, Amersham Biosciences, Little Chalfont, Bucks., U.K.) Western-blot analysis.

Determination of the kinetics of wild-type and mutant TK proteins for thymidine

Proteins of TK and mutants, which were expressed by pRsetA vector, had the histidine-tagged polypeptides at the N-terminus. The batch histidine beads' purification was used for TK protein purification according to the manufacturer's instruction (Qiagen, Hilden, Germany). The purification tube was prepared by loading 2 ml of Ni^{2+}-nitrilotriacetate agarose and washed with loading buffer (0.5 M NaCl/20 mM Tris/HCl, pH 7.9/10 % Nonidet P40) and rotated for 10 min. All subsequent steps were performed at 4 °C. The crude lysate was clarified by centrifugation at 6000 *g* for 30 min and loaded on to a purification tube. After

© 2004 Biochemical Society

BEST AVAILABLE COPY

overnight incubation, the tube was washed with two washing buffers (loading buffer plus 30 and 60 μM imidazole respectively) each for 10 min. The protein was obtained by incubating three times each with 1 ml of elution buffers (loading buffer plus 100, 200 and 300 μM imidazole respectively) for 20 min. Purified proteins were assessed on Coomassie Blue-stained gel and the protein concentration was quantitated with bicinchoninic acid kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and was stored at -70 °C for determining the K_m value.

To determine the K_m values of the wild-type and mutant EBV TKs for thymidine, reaction conditions were similar to that for the TK activity assay described above. [^{3}H]Thymidine concentrations varied from 0.5 to 2.25 μM. Lineweaver–Burk plots were used to determine the K_m and K_{cat} (V_{max}/[E]) values.

Metal-ion usage and substrate-competition assay

Metal-ion usage and substrate competition assays were performed using a procedure similar to that used for the TK activity assay. Several metal ions, including magnesium, manganese and zinc, were tested for their effect on enzyme activity. Assay conditions were 0.16 M Tris/HCl (pH 7.5), 0.14 mM albumin, 12.6 mM phosphocreatine, 11.2 units/ml creatine kinase, 120 μM thymidine, 2.35 μM [^{3}H]thymidine (6.7 Ci/mmol), 2.4 mM ATP, 9 mM NaF, 1.9 mM DTT and 2.4 mM metal chloride. Conditions of the substrate competition assay were the same as that for the TK activity assay except that the competitor was added to the reaction mixture to a 50-fold excess over the total thymidine concentration before adding the radioactive thymidine.

Western-blot analysis

The crude bacterial lysate was diluted 1:70 with loading buffer, electrophoresed by SDS/PAGE, blotted on to Immobilon-P (45 μm; Millipore, Bedford, MA, U.S.A.), and blocked with 10 mM Tris/HCl (pH 7.4), 0.9% NaCl and 4% (w/v) skim milk (blocking buffer) for 1.5 h. Anti-TK monoclonal antibody, 5F4C (Y.-R. Chang, T.-Y. Hsu and J.-Y. Chen, unpublished work), was used as the primary antibody and it was allowed to react for 1 h at room temperature (25 °C). The blot was washed and incubated with 1:5000 diluted, horseradish peroxidase-labelled goat anti-mouse antibody (Amersham Biosciences) at room temperature for 1 h. After incubation, the blot was washed three times in washing buffer, then developed with freshly prepared substrate for 1 min (ECL® Western blotting; Amersham Biosciences). The luminescence was detected by a short exposure to X-ray film. The relative concentration of each protein was determined by densitometry (UltraScan XL; Amersham Biosciences).

Phosphate transfer assay to determine the enzyme kinetics

Phosphate transfer assay was performed as described by Eriksson et al. [39] to determine the enzyme kinetics. Purified TK proteins were mixed with 1.6 μM [γ-^{32}P]ATP (6000 Ci/mmol), 50 mM Tris/HCl (pH 7.6), 0.5 mM $MgCl_2$, 100 mM KCl, 0.5 mg/ml BSA and 0.2–100 μM nucleoside in a final volume of 50 μl. The reaction mixture was incubated at 37 °C for 30 min; the reaction was terminated by boiling for 1 min. The mixture was centrifuged and 2–4 μl of the supernatant was spotted on a poly(ethyleneimine)–cellulose F (Merck, Darmstadt, Germany) thin layer sheet. Chromatography was performed for 8–12 h using 99% isobutyric acid/NH_4OH/water (66:1:33, by vol.) as the mobile phase. Products of the kinase reaction were detected by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The intensity of spots was determined by ImageQuant. Lineweaver–Burk plots were used to determine the enzyme kinetics.

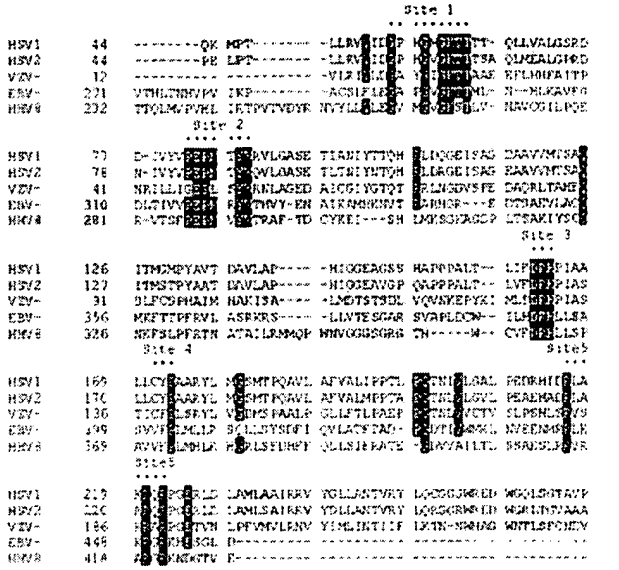


Figure 1 Alignment of five human herpesvirus TKs

Multiple alignment of the amino acid sequence of five human herpesvirus TKs, including α-herpesviruses HSV-1, HSV-2, VZV and γ-herpesviruses EBV, HHV-8, using Biology Workbench 3.0. Black blocks indicate amino acid identity. Five conserved regions are noted.

RESULTS

Alignment of five human herpesvirus TKs

We aligned the amino acid sequences of five human herpesvirus TKs: the α-herpesviruses HSV-1, HSV-2, VZV, γ-herpesviruses EBV and HHV-8 (human herpes virus-8), using Workbench 3.0 to identify conserved regions that might indicate functional domains. As shown in Figure 1, there are five conserved regions in these TK protein sequences. Among them, sites 3 and 4 are highly conserved and have been suggested to be involved in nucleoside binding through studies of crystal structure and mutation analysis of HSV-1 TK [32–36]. Site 3, consisting of the motif -DRH-, comprises three hydrophilic residues. The arginine at position 393 (Arg^{393}) is highly conserved among all TKs (the single amino acid and its position in the EBV TK was designated as Arg^{393}), suggesting its importance in the evolution of herpesviral TKs [40]. This site is important for TK activity and has been suggested to be involved in nucleoside recognition [32,35]. Site 4 comprises residues -(C/V)(Y/F)P- and is -CYP- in HSV-1 and HSV-2, -CFP- in VZV and -VFP- in EBV and HHV-8. Although site 4 is not as well conserved as site 3, it was still expected to participate in thymidine binding [33,36]. In the present study, Asp^{392}, Arg^{393} and His^{394} in site 3 and Val^{401} and Phe^{402} in site 4, which are predicted to be nucleoside-binding residues in EBV TK, were chosen for the investigation of their functional and biochemical roles.

TK activities of the Asp^{392} mutants

To investigate the putative role of Asp^{392} at site 3, a series of mutants were constructed, including D392E, D392H and D392N (Figure 2a). Protein expression was confirmed by Western blotting with EBV TK monoclonal antibody, and the activities of the

© 2004 Biochemical Society

BEST AVAILABLE COPY

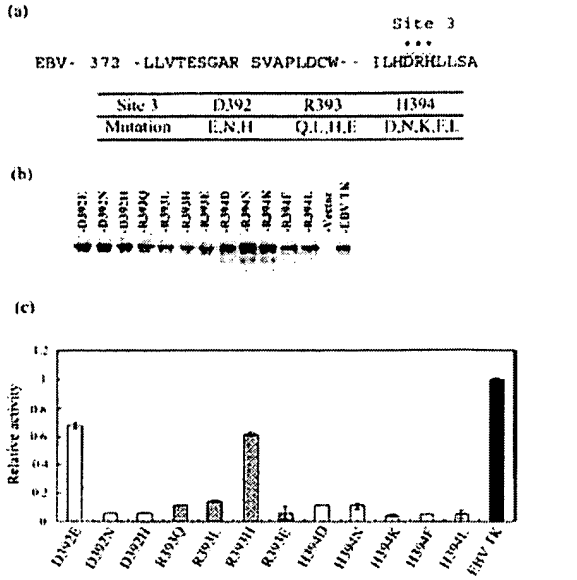


Figure 2 Mutational analysis of amino acids in the thymidine recognition site

(**a**) Mutations created within the conserved domain site 3 are indicated by * and blocked. (**b**) Protein expression in *E. coli* BL21(DE3)pLysS, transformed with plasmids D392E, D392N, D392H, R393Q, R393L, R393H, R393E, H394D, H394N, H394K, H394F, H394L, vector control and wild-type EBV TK, was detected using a mouse anti-TK monoclonal antibody 5F4C. (**c**) The relative TK activity was measured using [^{3}H]thymidine as the substrate. D392E and R393H retained approx. 60–70 % of the activity and the remaining mutants were inactive. The value of each mutant was normalized with the amount of relative expression in (**b**). Bars represent S.D. from duplicate assays.

Table 2 Kinetic parameters for thymidine of wild-type TK and site 3 and site 4 mutants

EBV TK proteins	K_m (μM)	K_{cat} (s^{-1})	Mean relative K_{cat}/K_m
Wild-type	4.83 ± 0.48	0.066 ± 0.001	1.00
D392E	22.9 ± 4.99	0.043 ± 0.018	0.13
R393H	17.9 ± 1.63	0.022 ± 0.001	0.09
V401C	11.3 ± 0.81	–	–
F402S	9.25 ± 0.24	0.044 ± 0.006	0.35
F402Y	8.77 ± 0.31	0.077 ± 0.011	0.63

–, not determined.

mutants were determined by TK activity assay. As shown in Figure 2(c), D392E retained 68 % activity of the wild-type enzyme but D392H and D392N lost almost all of their activities. The function of Asp^{392} could be compensated for by replacement with the negatively charged glutamic residue but not with the positively charged histidine residue, or the uncharged asparagine residue. Results indicate that the negative charge plays an important role at this position. The K_m and K_{cat} values (catalytic-centre activity) for thymidine of D392E were compared with that of the wild-type TK (Table 2). The K_m value for thymidine was 22.9 and 4.83 μM for the D392E mutant and wild-type TK respectively. The increased K_m value indicated that the mutant has decreased affinity for thymidine. The decreased K_{cat} and relative K_{cat}/K_m values may reflect local structure perturbation caused by the substituted residue, which interferes with the catalytic process. The results suggest that Asp^{392} is involved in nucleoside binding.

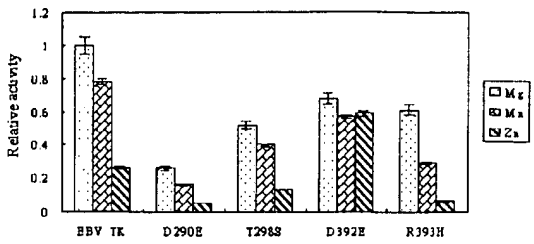


Figure 3 Metal-ion usage by the wild-type EBV TK and its mutants

Three metal ions, magnesium, manganese and zinc, were tested in the activity assays of EBV TK, D392E and R393H respectively. D290E and T298S, which were the mutants corresponding to the ATP-binding site of HSV-1 TK, were tested as controls. Relative activity was determined by comparing the value with wild-type TK in the presence of magnesium. Bars represent S.D. from duplicate assays.

TK activities of Arg^{393} mutants

The involvement of Arg^{393} residue in TK activity was tested by replacing it with a glutamine, leucine, histidine or glutamic residue (Figure 2a). Protein expression was confirmed by Western blotting with EBV TK monoclonal antibody (Figure 2b). TK activities were measured and calculated as shown in Figure 2(c). The R393H mutant, in which arginine was replaced by the positively charged histidine residue, retained 61 % activity relative to that of the wild-type. However, substitution of Arg^{393} residue with leucine, glutamine or aspartic residues, which are hydrophobic, uncharged and negatively charged residues respectively resulted in loss of activity. These results suggested that a positive charge may be required to maintain the functional activity of EBV TK. Furthermore, the K_m and K_{cat} values for thymidine of the R393H mutant were determined (Table 2). The K_m value for thymidine was 17.9 μM, a 4-fold increase, and the K_{cat} value was reduced to one-third, resulting in a marked decrease in the K_{cat}/K_m values in comparison with that of the wild-type enzyme. These results revealed that Arg^{393} is also involved in nucleoside binding.

TK activities of the His^{394} mutants

His^{394} was also found to be highly conserved in site 3 (Figure 1), even though its function was not very clear. His^{394} was replaced with asparagine, lysine, phenylalanine, leucine or aspartic residue (Figure 2a). As shown in Figure 2(b), the TK protein expression was confirmed by Western blotting and all mutants lost their activities (Figure 2c). Since all the His^{394} mutants, regardless of charge and structure, lost their enzymic activity, it seems that His^{394} plays a crucial role in enzymic activity.

Metal-ion usage of the site 3 mutants

An amino acid in adenylate kinase equivalent to EBV Asp^{392} was shown to fix Mg^{2+} via water molecules [41]. For HSV-1 TK, replacement of this aspartic residue with glutamine residue did not alter the requirement for a bivalent cation [42], but structural studies revealed that the negative charge of this aspartic residue may be important for co-ordination with a bivalent cation [35,36]. To evaluate the role of the Asp^{392} in site 3 further, the metal-ion usage was assayed. Three metal ions, Mg^{2+}, Mn^{2+} and Zn^{2+}, were tested with D392E, R393H and wild-type TKs. D290E and T298S, which are in site 1, have been suggested to be involved in ATP binding and were tested as controls. Results were similar to those reported by Tung and Summers [27], Mg^{2+} is the preferred ion for EBV TK, Mn^{2+} is less favoured and Zn^{2+} could inhibit its activity, as shown in Figure 3. Among the mutants tested,

© 2004 Biochemical Society

BEST AVAILABLE COPY

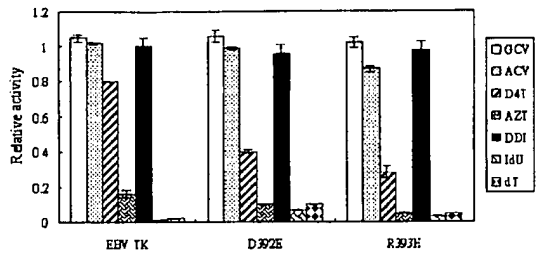


Figure 4 Determination of the substrate specificity of site 3 mutants

GCV, ACV, D4T, AZT, DDI and IdU were used at 50-fold excess over the total thymidine concentration to compete for the binding of thymidine by wild-type TK, D392E and R393H. Relative activity was determined by comparing the value with wild-type TK in the absence of the competitors. Bars represent S.D. from duplicate assays.

only D392E revealed an altered pattern of metal-ion usage, where nearly similar activities were found for all three metal ions. These results show that, in addition to its involvement in enzymic activity and nucleoside binding, Asp^{392} also plays an important role in metal-ion binding.

Substrate specificity of the site 3 mutants

The importance of site 3 of the EBV TK in the nucleoside and metal-ion binding was deduced from the above results. We performed competition assays to determine the substrate specificities of the site 3 mutants. Six commonly used nucleoside analogues, GCV, ACV, AZT (3′-azido-3′-deoxythymidine), D4T (3′-deoxy-2′,3′-didehydrothymidine), DDI (dideoxyinosine) and IdU (iododeoxyuridine), were chosen and thymidine was used as the control. These drugs were used at a 50-fold higher concentration when compared with that of thymidine to compete for binding. With wild-type EBV TK, thymidine usage could not be competed by ACV, GCV and DDI but could be competed partially by D4T, and almost completely by AZT and IdU (Figure 4). This result is similar to other studies, suggesting that EBV TK seems to favour pyrimidine nucleoside analogues such as D4T, AZT and IdU, but not purine nucleoside analogues such as ACV, GCV and DDI [43]. With the mutants D392E and R393H (Figure 4), similar to the wild-type, thymidine could not be competed by ACV, GCV and DDI but both mutants were competed for their activity by AZT and IdU. Interestingly, for both mutants, more than 50% of the activity was competed by D4T, suggesting that they had higher affinities for this nucleoside analogue when compared with the wild-type.

To address this phenomenon further, the IC_{50} and the kinetic parameters of wild-type TK and mutants for GCV, AZT and D4T were determined (Table 3). The K_m values for D4T of D392E and R393H were slightly decreased when compared with that of wild-type. Both K_{cat} and K_{cat}/K_m values of D392E and R393H for D4T were increased, reflecting that their catalytic efficiency for D4T was better when compared with wild-type enzyme. On the basis of these results, the residues at site 3 were supposed to play a role in the determination of substrate specificity.

TK activities of the Val^{401} mutants

Substitution of Cys^{171} in HSV-1 TK (corresponding to EBV TK Val^{401}) with a glycine residue did not destroy its enzymic activity [44]. It was suggested that this residue is not involved in nucleoside binding and is not essential for the catalytic activity of the enzyme. No specific role could be assigned to this residue from

Table 3 Kinetic parameters for nucleoside analogues of wild-type TK and site 3 and site 4 mutants

EBV TK proteins	Nucleoside drugs	Kinetic parameters IC_{50} (μM)	K_m (μM)	K_{cat} (s^{-1})	Mean K_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
TK	GCV	> 200	> 100	–	–
	AZT	8.38 ± 0.47	3.35 ± 0.07	0.034 ± 0.002	0.01038
	D4T	151 ± 8.5	6.97 ± 0.75	0.036 ± 0.007	0.00517
D392E	GCV	171.5 ± 29	> 100	–	–
	AZT	6.12 ± 0.78	4.25 ± 0.21	0.044 ± 0.026	0.01011
	D4T	16.2 ± 0.49	5.67 ± 0.64	0.075 ± 0.011	0.01039
R292H	GCV	153 ± 37.5	–	–	–
	AZT	5.58 ± 0.06	3.30 ± 1.27	0.056 ± 0.032	0.01622
	D4T	13.6 ± 0.92	4.57 ± 0.45	0.075 ± 0.046	0.01600
F402S	GCV	> 200	–	–	–
	AZT	84.8 ± 0.21	63.3 ± 2.40	0.020 ± 0.032	0.00059
	D4T	101 ± 5.66	60.5 ± 27.6	0.030 ± 0.009	0.00060

–, not determined.

three-dimensional studies of the HSV-1 TK [34,36]. To determine the functional roles of the amino acid residues at site 4 of the EBV TK, five mutants were constructed: V401C, V401L, V401S, F402S and F402Y (Figure 5a). Three of Val^{401} mutants, those substituted with the hydrophilic amino acids cysteine or serine or the hydrophobic leucine, were used to determine the contribution of this residue to EBV TK activity. The activities of the mutants were determined after confirming the expression level of each protein (Figure 5b). The mutant V401C retained approx. 80% of the wild-type activity, whereas the V401L and V401S mutants were almost inactive (Figure 5c). EBV TK has a valine residue at this position, whereas HSV TK has a cysteine residue; therefore it is not surprising that the mutant V401C was active. However, EBV TK was inactive when this residue was changed to the hydrophilic residue serine. Similarly, the HSV-1 TK activity was lost when the equivalent residue was changed to serine [44]. The kinetic parameters of V401C mutant for thymidine were also different from that of the wild-type TK (Table 2). These findings suggest that Val^{401} in site 4 of the EBV TK may play some role in its activity and requires further study.

TK activities of the Phe^{402} mutants

The importance of this residue in HSV-1 TK was discussed extensively, especially the three-dimensional structural studies [34,36]. In a random mutational analysis, it was found that Tyr^{172} of HSV-1 TK (corresponding to EBV TK Phe^{402}) could be replaced only by phenylalanine, without changing its activity [33]. The three-dimensional crystal structure of the HSV-1 TK also revealed that this residue is stacking on the thymidine and contacts it closely [36]. Two mutants were designed at this position, F402S and F402Y. After expression, the amounts of the mutant proteins and enzyme activities were determined (Figure 5b). As shown in Figure 5(c), the mutant F402Y was fully active but F402S was partially inactive. The F402Y had a 122% relative activity when compared with that of wild-type, whereas substitution with a serine residue retained 60% of the wild-type activity. These results revealed that the aromatic ring of phenylalanine and tyrosine residues at this position is important, although serine residue, without an aromatic ring in its structure, could compensate this function partially. The K_m values of these two mutants altered slightly and K_{cat}/K_m values were decreased

© 2004 Biochemical Society

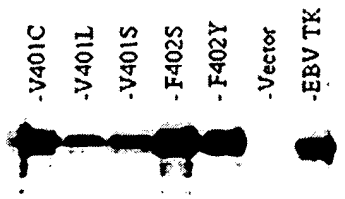
BEST AVAILABLE COPY

(a)

Site4
***
EBV- 399 SVVFPLMLLR SQLLSYSDFI

Site4	V401	F402
Mutation	C, L, S	S, Y

(b)



(c)

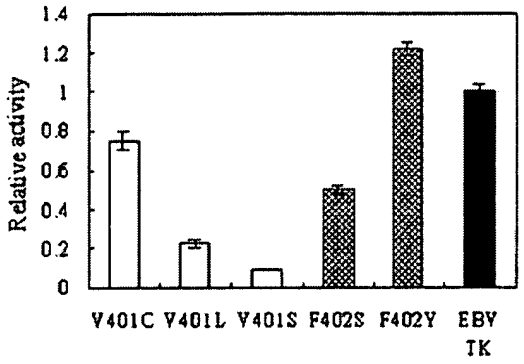


Figure 5 Mutational analysis of amino acids in the thymidine-binding site

(**a**) Mutations created within conserved domain site 4 are indicated by * and blocked. (**b**) Protein expression in *E. coli* BL21(DE3)pLysS, transformed with plasmids containing V401C, V401L, V401S, F402S, F402Y, vector control and wild-type EBV TK, was detected with mouse anti-TK monoclonal antibody 5F4C. (**c**) The relative activity was measured using [^{3}H]thymidine as the substrate. Val^{401} mutants varied in their activities. Phe^{402} mutants retained at least 60 % of the wild-type activity. The value for each mutant was normalized with the relative expression amount in (**b**). Bars represent S.D. from duplicate assays.

(Table 2), reflecting that Phe^{402} has a decreased catalytic efficiency and it also plays a role in nucleoside binding.

Substrate specificity of the site 4 mutants

To determine further the contribution of the amino acid residues at site 4 to substrate specificity, six commonly used nucleoside analogues were prepared as described in the Experimental section. The competition assay was performed and the results are shown in Figure 6. F402S altered the substrate specificity, whereas F402Y had the same specificity as the wild-type. For F402S, not only the purine, but also the pyrimidine analogues D4T, AZT and IdU, were much less efficient in competing with thymidine for binding. The significant difference in K_m and K_{cat}/K_m values for AZT and D4T indicated that the binding efficiency of this mutant to these drugs was affected severely (Table 3). It strongly suggested that Phe^{402} is important in substrate selection.

Recently developed L-form nucleoside analogues L-FMAU and L-I-OddU have been shown to inhibit EBV replication [10]. To confirm further the role of site 4 in substrate usage, these two

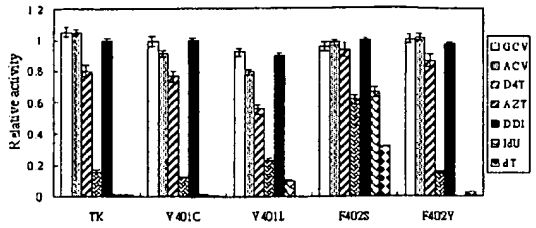


Figure 6 Determination of substrate specificity of site 4 mutants

GCV, ACV, D4T, AZT, DDI and IdU were used at 50-fold excess over the total thymidine concentration to compete for the binding of thymidine by wild-type TK, V401C, V401L, F402S and F402Y. Unlabelled thymidine was used as a control. Relative activity was determined by comparing the value with wild-type TK in the absence of the competitors. Bars represent S.D. from duplicate assays.

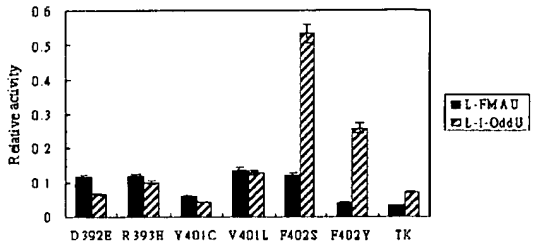


Figure 7 Competition of L-form nucleoside analogues to thymidine in site 3 and site 4 mutants

L-FMAU and L-I-OddU were used at 50-fold excess over total thymidine concentration to compete for binding of thymidine by wild-type TK and site 3 and site 4 mutants. Relative activity was determined by comparing the values with that in the presence of DMSO. Bars represent S.D. from duplicate assays.

nucleoside analogues were also used to compete with thymidine for binding at sites 3 and 4. As shown in Figure 7, these two nucleoside analogues competed well with thymidine for the wild-type TK. This result is consistent with the reported results [10]. The competitive effects were also observed for site 3 and site 4 mutants, except for F402S and F402Y. Surprisingly, L-I-OddU was relatively incapable of competing with thymidine for binding, indicating that Phe^{402} is critical in substrate selection.

DISCUSSION

Herpesviral TKs have been chosen as important targets for the development of anti-viral agents and gene therapies. Accordingly, EBV TK is considered a potential target for the treatment of EBV-associated disease and as a tool for anticancer therapies [9,24,26,45]. Understanding the structure–function relationship of TK enzymes is helpful in designing new antiviral drugs and gene therapy strategies. Site-directed mutagenesis, in combination with crystal structure studies, has provided significant insights in this direction. However, there are few studies on the structure–function relationship of EBV TK. To the best of our knowledge, this is the first study to describe the importance of individual amino acids in the conserved regions of EBV TK. In the absence of any structural information, we determined the nucleoside-binding sites and examined the roles of amino acid residues at these sites using site-directed mutagenesis. Following the alignment of the TK sequences of five human herpesviruses and comparing with the studies of HSV-1 TK, our results confirmed that sites 3 and 4 of EBV TK are involved in nucleoside binding, metal-ion binding and substrate specificity.

© 2004 Biochemical Society

Our results indicated that Asp^{392} plays multiple roles in site 3 of EBV TK. From structural information for HSV-1 TK, the corresponding Asp^{162} is important for substrate and metal-ion binding. Results obtained from other studies suggested that it fixes the Mg^{2+} ion via a water molecule through its negative charge, in addition to binding to the phospho group of TMP [35,36]. However, this function was not supported by another study [42], which showed that the requirement for a bivalent cation was not altered when it was changed to an uncharged glutamine residue. In our study, the metal-ion usage of all site 3 and site 4 mutants is similar to that of HSV-1 TK [46]. The catalytic activity of the bivalent cations tested for wild-type EBV TK was in the order $Mg^{2+} > Mn^{2+} > Zn^{2+}$, where, in the presence of Zn^{2+}, the enzyme is inactive. Interestingly, only the D392E substitution changed the usage pattern of metal ions, especially for Zn^{2+}, with which it exhibited the same activity as with Mg^{2+} and Mn^{2+} (Figure 3). Furthermore, the concentration of Mg^{2+} required for activity was much lower when compared with that of the wild-type (results not shown).

Arg^{393}, similar to its corresponding amino acid of HSV-1 TK, Arg^{163} [32], was found to be crucial for the activity. According to the models of adenylate kinase and HSV-1 TK, this arginine residue is in a strained conformation and forms a hydrogen bond with the phosphate groups of AMP and TMP [35,36,41]. Further evidence of a functional role was provided by Sawyer et al. [47], who identified a substitution with glutamine at this position in an ACV-resistant virus, corroborating the role of this arginine residue in nucleoside binding and substrate specificity. The increase in K_m value for thymidine (Table 2) and binding affinity for D4T (Figure 4, Table 3) of the R393H mutant revealed that this arginine residue participates in nucleoside binding and substrate specificity, at least for D4T. The potential role of R393H TK in the nucleoside utilization is under further investigation.

His^{394} is a very important residue and cannot be substituted with other amino acids with different properties. By analogy, Black and Loeb [32] also found that the histidine residue at the corresponding position in HSV-1 TK could not be substituted with other amino acids. On the basis of the structural information of HSV-1 TK, it seems probable that this histidine contributes to the special conformation for a functional TK activity, even though the precise role remains unclear [34–36].

It is clear that Phe^{402} plays an important role at site 4. Our results reveal that the special structures of phenylalanine and tyrosine residues are important for the activity of EBV TK. This finding was consistent with the study [32] that activity was retained only with phenylalanine at Tyr^{172} of HSV-1 TK. On the basis of the information obtained from the density functional studies on HSV-1 TK, the substrate–Tyr^{172} interaction is essentially an electrostatic force and was suggested to be involved in the substrate binding [48]. The three-dimensional structure of the HSV-1 TK showed the aromatic ring of the tyrosine residue at this position stacked on to the thymine ring, and Arg^{163} (HSV-1 TK) formed the hydrogen bond with the hydroxy group of the tyrosine residue at this position [36]. Interestingly, F402Y of the EBV TK had higher activity when compared with that of the wild-type TK (Figure 5c). This phenomenon suggests that the hydrogen bond is important to fix thymidine, because the phenylalanine residue at this position in EBV TK could not form the hydrogen bonds with Arg^{393}.

There are differences between EBV and HSV-1 TKs in their substrate specificities, which may have resulted from the divergence of protein sequences. HSV-1 TK can phosphorylate purine nucleoside analogues such as GCV, ACV and DDI, and pyrimidine nucleoside analogues such as D4T, AZT and IdU, whereas EBV TK prefers to utilize pyrimidine nucleoside analogues only [43,49]. Our results agreed with these findings (Figures 4 and 6).

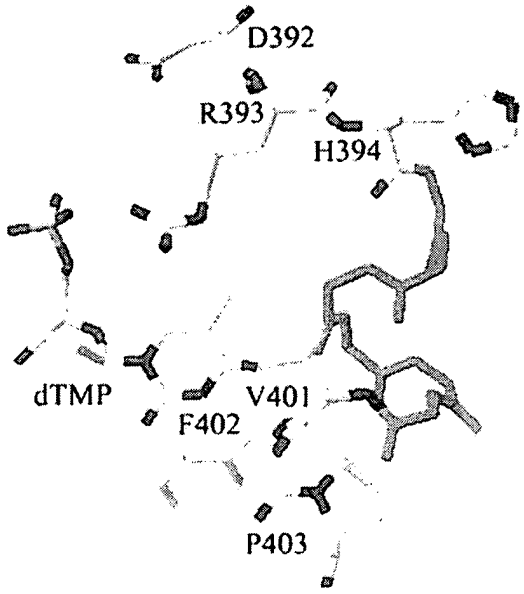


Figure 8 Hypothetical model for the structure of the conserved nucleoside-binding region of EBV TK

This model is based on the structure for the nucleoside-binding region of HSV-1 TK. The position and geometry of dTMP and amino acids in the conserved nucleoside-binding sites are shown as labelled. The polypeptide shown in light blue is the non-conserved region. The co-ordinate template was indexed as 2VTK in the Protein Data Bank. Structural modelling exploration was conducted using the program Swiss-PdbViewer v.3.6b3.

Interestingly, comparison of the TK protein sequences of these human herpesviruses enables their separation into two types; HSV-1 and HSV-2 in one class and EBV and HHV-8 in the other. For HSV-1 and HSV-2, not only are the residues of the conserved nucleoside-binding sites completely identical, but the substrate diversity is also almost the same. For the conserved nucleoside binding sites 3 and 4, all herpesviruses have the same residues (-DRH-) in site 3, whereas EBV and HHV-8 both have -VFP- in site 4. They also prefer to utilize the pyrimidine analogues [50]. Furthermore, it is reasonable to speculate that Phe^{402} may be involved in determining the difference between HSV-1 and EBV TK in substrate range.

In Figure 8, a model is proposed for the protein structure of the nucleoside-binding region spanning from site 3 to site 4 in EBV TK, based on structural information for HSV-1 TK [34,36]. We can find relevant residues forming the cavity to accommodate the nucleoside substrate for catalysis. At site 3, Asp^{392} may provide its negative charge to fix the Mg^{2+} and phosphate group of dTMP through water molecules and Arg^{393} provides its positive charge to fix the phosphate of TMP. At site 4, Phe^{402} stacks on the thymine ring to stabilize the substrate. Some other residues, which are not located in the conserved sites 3 and 4, may also contribute and co-operate with the conserved residues to execute the catalytic process.

EBV TK, similar to HSV-1 TK, has been proved sensitive to many nucleoside analogues, implying that it can be applied in gene therapy [9,43]. Although HSV-1 TK has broader substrate specificity, EBV TK has a specific advantage in using anti-HIV drugs, such as AZT and D4T, which may have an extra benefit in treatment of AIDS-associated cancer [43]. On the other hand, our results may also be applied in other herpesvirus TKs, to gain a useful mutant. Our next goal will be to create double or triple

© 2004 Biochemical Society

mutations in these two regions to find a mutant with better TK activity and broader substrate range for usage *in vivo*.

While we try to build a plausible model by analogy with other TKs, the precise interpretation of these results, as well as the effects of the mutants, await a detailed structural analysis of EBV TK. We expect these results to provide information useful in understanding the enzymic mechanism and for application in the development of new antiviral drugs and creation of an effective tool for gene therapy.

We thank Dr Y.-C. Cheng for kindly providing nucleoside analogues L-FMAU and L-I-OddU. We appreciate Dr T. J. Harrison (Royal Free and University College Medical School, University College London, London, U.K.) and Dr Z.-F. Chang (Department of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China) for critical reviews and comments on the paper. C.-C. W. was supported by grants from the National Science Council (NSC 85-2331-B-002-187-M06, NSC 86-2314-B-002-114, NSC 87-2314-B-002-234, NSC 88-2312-B-002-005, NSC 89-2320-B-002-001, NSC 89-2320-B-002-202, NSC 90-2320-B-002-159 and NSC 91-2320-B-002-100).

REFERENCES

1 Jamieson, A. T., Gentry, G. A. and Subak-Sharpe, J. H. (1974) Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. **24**, 465–480
2 Colby, B. M., Shaw, J. E., Elion, G. B. and Pagano, J. S. (1980) Effect of acyclovir [9-(2-hydroxyethoxymethyl)guanine] on Epstein–Barr virus DNA replication. J. Virol. **34**, 560–568
3 Cheng, Y. C., Huang, E. S., Lin, J. C., Mar, E. C., Pagano, J. S., Dutschman, G. E. and Grill, S. P. (1983) Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine against herpesvirus *in vitro* and its mode of action against herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. **80**, 2767–2770
4 Furman, P. A., McGuirt, P. V., Keller, P. M., Fyfe, J. A. and Elion, G. B. (1980) Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Virology **102**, 420–430
5 St Clair, M. H., Lambe, C. U. and Furman, P. A. (1987) Inhibition by ganciclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Antimicrob. Agents Chemother. **31**, 844–849
6 Culver, K. W., van Gilder, J., Link, C. J., Carlstrom, T., Buroker, T., Yuh, W., Koch, K., Schabold, K., Doornbas, S. and Wetjen, B. (1994) Gene therapy for the treatment of malignant brain tumors with *in vivo* tumor transduction with the herpes simplex thymidine kinase gene/ganciclovir system. Hum. Gene Ther. **5**, 343–379
7 Grignet-Debrus, C. and Calberg-Bacq, C. M. (1997) Potential of Varicella Zoster virus thymidine kinase as a suicide gene in breast cancer cells. Gene Ther. **4**, 560–569
8 Loubiere, L., Tiraby, M., Cazaux, C., Brisson, E., Grisoni, M., Zhao-Emonet, J., Tiraby, G. and Klatzmann, D. (1999) The equine herpes virus 4 thymidine kinase is a better suicide gene than the human herpes virus 1 thymidine kinase. Gene Ther. **6**, 1638–1642
9 Moore, S. M., Cannon, J. S., Tanhehco, Y. C., Hamzeh, F. M. and Ambinder, R. F. (2001) Induction of Epstein–Barr virus kinases to sensitize tumor cells to nucleoside analogues. Antimicrob. Agents Chemother. **45**, 2082–2091
10 Kira, T., Grill, S. P., Dutschman, G. E., Lin, J. S., Qu, F., Choi, Y., Chu, C. K. and Cheng, Y. C. (2000) Anti-Epstein–Barr virus (EBV) activity of β-L-5-iododioxolane uracil is dependent on EBV thymidine kinase. Antimicrob. Agents Chemother. **44**, 3278–3284
11 Pilger, B. D., Perozzo, R., Alber, F., Wurth, C., Folkers, G. and Scapozza, L. (1999) Substrate diversity of herpes simplex virus thymidine kinase. Impact of the kinematics of the enzyme. J. Biol. Chem. **274**, 31967–31973
12 Henle, G., Henle, W. and Diehl, V. (1968) Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc. Natl. Acad. Sci. U.S.A. **59**, 94–101
13 Evans, A. S., Niederman, J. C. and McCollum, R. W. (1968) Seroepidemiologic studies of infectious mononucleosis with EB virus. N. Engl. J. Med. **279**, 1121–1127
14 Henle, W., Henle, G. E. and Horwitz, C. A. (1974) Epstein–Barr virus specific diagnostic tests in infectious mononucleosis. Hum. Pathol. **5**, 551–565
15 Greenspan, D., De Souza, Y. G., Conant, M. A., Hollander, H., Chapman, S. K., Lennette, E. T., Petersen, V. and Greenspan, J. S. (1990) Efficacy of desciclovir in the treatment of Epstein–Barr virus infection in oral hairy leukoplakia. J. Acquir. Immune Defic. Syndr. **3**, 571–578
16 Greenspan, J. S., Greenspan, D., Lennette, E. T., Abrams, D. I., Conant, M. A., Petersen, V. and Freese,U.K. (1985) Replication of Epstein–Barr virus within the epithelial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion. N. Engl. J. Med. **313**, 1564–1571
17 Webster-Cyriaque, J. and Raab-Traub, N. (1998) Transcription of Epstein–Barr virus latent cycle genes in oral hairy leukoplakia. Virology **248**, 53–65
18 Epstein, M. A., Achong, B. G. and Barr, Y. M. (1964) Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet **1**, 702–703
19 de-The, G., Geser, A., Day, N. E., Tukei, P. M., Williams, E. H., Beri, D. P., Smith, P. G., Dean, A. G., Bronkamm, G. W., Feorino, P. et al. (1978) Epidemiological evidence for causal relationship between Epstein–Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature (London) **274**, 756–761
20 Old, J. L., Boyse, E. A., Oettgen, H. F., de Harven, E., Geering, G., Williamson, B. and Clifford, P. (1966) Precipitating antibody in human serum to an antigen present in cultured Burkitt's lymphoma cells. Proc. Natl. Acad. Sci. U.S.A. **56**, 1699–1704
21 Su, I. J. and Hsieh, H. C. (1992) Clinicopathological spectrum of Epstein–Barr virus-associated T cell malignancies. Leuk. Lymphoma **7**, 47–53
22 Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C. et al. (1984) DNA sequence and expression of the B95-8 Epstein–Barr virus genome. Nature (London) **310**, 207–211
23 Littler, E., Zeuthen, J., McBride, A. A., Trost Sorensen, E., Powell, K. L., Walsh-Arrand, J. E. and Arrand, J. R. (1986) Identification of an Epstein–Barr virus-coded thymidine kinase. EMBO J. **5**, 1959–1966
24 Mentzer, S. J., Fingeroth, J., Reilly, J. J., Perrine, S. P. and Faller, D. V. (1998) Arginine butyrate-induced susceptibility to ganciclovir in an Epstein–Barr-virus-associated lymphoma. Blood Cells Mol. Dis. **24**, 114–123
25 Faller, D. V., Mentzer, S. J. and Perrine, S. P. (2001) Induction of the Epstein–Barr virus thymidine kinase gene with concomitant nucleoside antivirals as a therapeutic strategy for Epstein–Barr virus-associated malignancies. Curr. Opin. Oncol. **13**, 360–367
26 Mentzer, S. J., Perrine, S. P. and Faller, D. V. (2001) Epstein–Barr virus post-transplant lymphoproliferative disease and virus-specific therapy: pharmacological re-activation of viral target genes with arginine butyrate. Transpl. Infect. Dis. **3**, 177–185
27 Tung, P. P. and Summers, W. C. (1994) Substrate specificity of Epstein–Barr virus thymidine kinase. Antimicrob. Agents Chemother. **38**, 2175–2179
28 Littler, E. and Arrand, J. R. (1988) Characterization of the Epstein–Barr virus-encoded thymidine kinase expressed in heterologous eucaryotic and procaryotic systems. J. Virol. **62**, 3892–3895
29 Hsu, T. Y., Liu, M. W., Chang, Y. R., Pai, C. Y., Liu, M. Y., Yang, C. S. and Chen, J. Y. (1996) Functional analysis of C-terminal deletion mutants of Epstein–Barr virus thymidine kinase. J. Gen. Virol. **77**, 1893–1899
30 Balasubramaniam, N. K., Veerisetty, V. and Gentry, G. A. (1990) Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation. J. Gen. Virol. **71**, 2979–2987
31 Liu, Q. Y. and Summers, W. C. (1988) Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. Virology **163**, 638–642
32 Black, M. E. and Loeb, L. A. (1993) Identification of important residues within the putative nucleoside binding site of HSV-1 thymidine kinase by random sequence selection: analysis of selected mutants *in vitro*. Biochemistry **32**, 11618–11626
33 Munir, K. M., French, D. C., Dube, D. K. and Loeb, L. A. (1992) Permissible amino acid substitutions within the putative nucleoside binding site of herpes simplex virus type 1 encoded thymidine kinase established by random sequence mutagenesis. J. Biol. Chem. **267**, 6584–6589
34 Brown, D. G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P. J., Melitz, C., Summers, W. C. and Sanderson, M. R. (1995) Crystal structures of the thymidine kinase from herpes simplex virus type-1 in complex with deoxythymidine and ganciclovir. Nat. Struct. Biol. **2**, 876–880
35 Wild, K., Bohner, T., Aubry, A., Folkers, G. and Schulz, G. E. (1995) The three-dimensional structure of thymidine kinase from herpes simplex virus type 1. FEBS Lett. **368**, 289–292
36 Wild, K., Thomas, B., Gerd, F. and George, S. (1997) The structure of thymidine kinase from herpes simplex virus type 1 in complex with substrate and a substrate analogue. Protein Sci. **6**, 2097–2106
37 Liu, M. Y., Pai, C. Y., Shieh, S. M., Hsu, T. Y., Chen, J. Y. and Yang, C. S. (1992) Cloning and expression of cDNA encoding the Epstein–Barr virus thymidine kinase gene. J. Virol. Methods **40**, 107–118
38 Hsu, T. Y., Pai, C. Y., Shieh, S. M., Cho, S. M., Liu, M. Y., Chen, J. Y. and Yang, C. S. (1992) Use of antigen expressed in bacteria for detection of EBV-specific thymidine kinase antibodies in sera from patients with nasopharyngeal carcinoma. J. Med. Virol. **38**, 214–219
39 Eriksson, S., Kierdaszuk, B., Munch-Petersen, B., Oberg, B. and Johansson, N. G. (1991) Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem. Biophys. Res. Commun. **176**, 586–592
40 Gentry, G. A. (1992) Viral thymidine kinases and their relatives. Pharmacol. Ther. **54**, 319–355

© 2004 Biochemical Society

41 Abele, U. and Schulz, G. E. (1995) High-resolution structures of adenylate kinase from yeast ligated with inhibitor Ap5A, showing the pathway of phosphoryl transfer. Protein Sci. **4**, 1262–1271
42 Black, M. E., Rechtin, T. M. and Drake, R. R. (1996) Effect on substrate binding of an alteration at the conserved aspartic acid-162 in herpes simplex virus type 1 thymidine kinase. J. Gen. Virol. **77**, 1521–1527
43 Cazaux, C., Tiraby, M., Loubiere, L., Hatren, L., Klatzmann, D. and Tiraby, G. (1998) Phosphorylation and cytotoxicity of therapeutic nucleoside analogues: a comparison of α and γ herpesvirus thymidine kinase suicide genes. Cancer Gene Ther. **5**, 83–91
44 Inglis, M. M. and Darby, G. (1987) Analysis of the role of the cysteine 171 residue in the activity of herpes simplex virus type 1 thymidine kinase by oligonucleotide-directed mutagenesis. J. Gen. Virol. **68**, 39–46
45 Westphal, E. M., Mauser, A., Swenson, J., Davis, M. G., Talarico, C. L. and Kenney, S. C. (1999) Induction of lytic Epstein–Barr virus (EBV) infection in EBV-associated malignancies using adenovirus vectors *in vitro* and *in vivo*. Cancer Res. **59**, 1485–1491
46 Cheng, Y. C. (1976) Deoxythymidine kinase induced in the HeLa TK-cells by herpes simplex virus type I and type II. Substrate specificity and kinetic behavior. Biochim. Biophys. Acta **452**, 370–381
47 Sawyer, M. H., Inchauspe, G., Biron, K. K., Waters, D. J., Straus, S. E. and Ostrove, J. M. (1988) Molecular analysis of the pyrimidine deoxyribonucleoside kinase gene of wild-type and acyclovir-resistant strains of Varicella Zoster virus. J. Gen. Virol. **69**, 2585–2593
48 Alber, F., Kuonen, O., Scapozza, L., Folkers, G. and Carloni, P. (1998) Density functional studies on herpes simplex virus type 1 thymidine kinase–substrate interactions: the role of Tyr-172 and Met-128 in thymine fixation. Proteins **31**, 453–459
49 Gustafson, E. A., Chillemi, A. C., Sage, D. R. and Fingeroth, J. D. (1998) The Epstein–Barr virus thymidine kinase does not phosphorylate ganciclovir or acyclovir and demonstrates a narrow substrate specificity compared to the herpes simplex virus type 1 thymidine kinase. Antimicrob. Agents Chemother. **42**, 2923–2931
50 Gustafson, E. A., Schinazi, R. F. and Fingeroth, J. D. (2000) Human herpesvirus 8 open reading frame 21 is a thymidine and thymidylate kinase of narrow substrate specificity that efficiently phosphorylates zidovudine but not ganciclovir. J. Virol. **74**, 684–692

Received 28 November 2003; accepted 5 January 2004
Published as BJ Immediate Publication 5 January 2004, DOI 10.1042/BJ20031832

© 2004 Biochemical Society